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Alterations in Conserved Kir Channel-PIP_2 Interactions Underlie Channelopathies

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Summary

Inwardly rectifying K^+ (Kir) channels are important regulators of resting membrane potential and cell excitability. The activity of Kir channels is critically dependent on the integrity of channel interactions with phosphatidylinositol 4,5-bisphosphate (PIP_2). Here we identify and characterize channel-PIP_2 interactions that are conserved among Kir family members. We find basic residues that interact with PIP_2, two of which have been associated with Andersen's and Bartter's syndromes. We show that several naturally occurring mutants decrease channel-PIP_2 interactions, leading to disease.

Introduction

The role of phosphatidylinositol 4,5-bisphosphate (PIP_2) as a membrane-delimited second messenger has recently been appreciated (Hilgemann, 1997; Hilgemann et al., 2001). PIP_2 has been shown to regulate the activity of a number of transporters and channels, among them both native and recombinant inwardly rectifying K^+ (Kir) channels (Fan and Makielski, 1997; Hilgemann and Ball, 1996; Huang et al., 1998; Sui et al., 1998), the inositol trisphosphate (IP_3) receptor (Lupu et al., 1998), mammalian rod cyclic nucleotide-gated channels (Hirose et al., 1999), the capsaicin receptor channel (Raucher et al., 2000), and transporters such as the sodium-calcium exchanger (Hilgemann and Ball, 1996). Moreover, anionic phospholipids, and PIP_2 in particular, have been shown to directly bind to proteins as diverse as phospholipases, kinases, cytoskeletal proteins, and Kir channels (Ferguson et al., 1995; Huang et al., 1998). In general, protein-PIP_2 interactions, including those with Kir channels, are electrostatic in nature, with varying levels of head-group specificity (Ferguson et al., 1995; Kavran et al., 1998).

Kir subfamilies have been shown to serve a variety of functions, ranging from the regulation of the resting membrane potential and the pacing of both cardiac myocytes and neurons to the pancreatic regulation of insulin secretion and renal K^+ transport (Hille, 2001). Modulation of channel activity by changes in PIP_2 levels in the plasma membrane represents a crucial aspect of regulation for channels in the Kir superfamily (e.g., Kobrinsky et al., 2000). Fifteen cDNAs encoding Kir channel subunits have been isolated. Kir polypeptides consist of ~300–500 amino acids, which share ~40% amino acid identity and have a common secondary structure of a cytoplasmic N terminus, two membrane-spanning domains (M1 and M2), one pore-forming region between M1 and M2, and a long cytoplasmic C terminus. Four such subunits form the ion-conducting pathway of Kir channels. Members of at least four of the seven known Kir subfamilies have been reported to be activated and regulated by phospholipids, and in particular by PIP_2. These are Kir1.1 (Huang et al., 1998; Liou et al., 1999), Kir2.1 (Huang et al., 1998; Soom et al., 2001; Zhang et al., 1999), Kir3.1, Kir3.2, Kir3.4 (Huang et al., 1998; Sui et al., 1998), Kir6.1, and Kir6.2 (Baukrowitz et al., 1998; Fan and Makielski, 1997; Hilgemann and Ball, 1996; Huang et al., 1998; Shyng and Nichols, 1998). For these channels, depletion of PIP_2 has been reported to cause run-down (disappearance) of channel activity. Direct application of PIP_2-containing liposomes to membrane patches reactivates run-down channels. Positively charged residues of Kir channels have been hypothesized to be tethered by the electrostatic forces of the anionic phospholipid head-groups at the cytoplasmic face of the membrane, maintaining channel activity (Fan and Makielski, 1997).

Kir channel-PIP_2 interactions are crucial for channel activity and regulation. Given that interactions of proteins with PIP_2 have been shown to be electrostatic in nature (Kavran et al., 1998), we set out to identify basic amino acids that affect channel-PIP_2 interactions in a conserved fashion among the Kir channels. We mutated conserved basic amino acids in both the N and C termini of Kir2.1. We identified 10 residues that show electrostatic interactions with PIP_2. Two of the residues that we found to be important for channel-PIP_2 interactions have been associated with Bartter's (Schulte et al., 1999) and Andersen's syndromes (Plaster et al., 2001). We show that other mutations associated with both the Andersen's and Bartter's syndromes are neighboring PIP_2 binding sites and allosterically decrease channel-PIP_2 interactions. Our results suggest that decrease in PIP_2-channel interactions is the mechanism underlying the loss-of-function phenotype for several mutations associated with both Andersen's and Bartter's syndromes. We also show that mutant channels associated with disease have increased susceptibility to modulation by stimuli that decrease membrane PIP_2 levels. This would lead to a further loss of channel function upon stimulation, exacerbating the disease phenotype.

Results

Kir2.1 Mutants Have Altered PIP_2Ab Inhibition Kinetics

Using as a model Kir2.1 (IRK1), which is known to interact strongly with PIP_2 (Huang et al., 1998; Zhang et al.,

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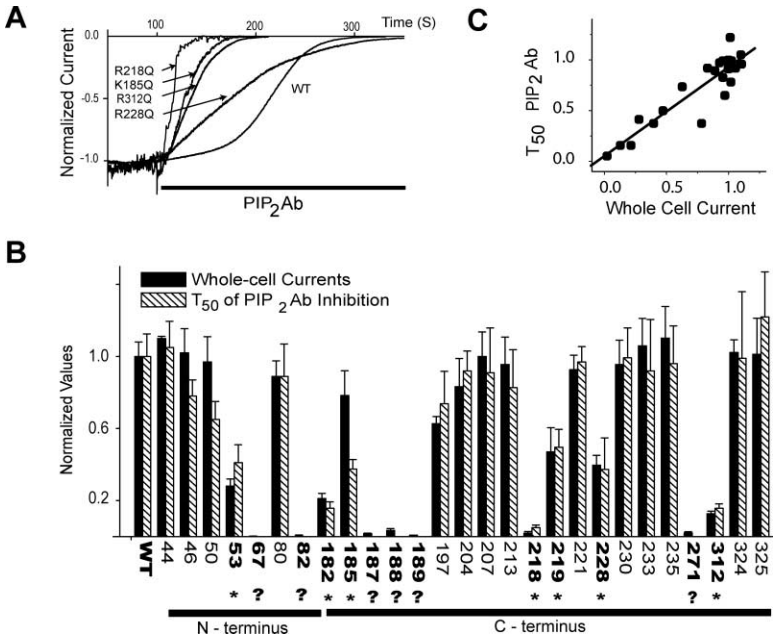

Figure 1. PIP_2Ab Inhibition of Kir2.1 Mutants

(A) Normalized current measured in inside-out patches for Kir2.1 wild-type and several of the point mutants studied at −80mV.

(B) Summary data (n = 4–7) of whole-cell current levels and T_{50} values are plotted for each of the Kir2.1 mutants. Asterisks denote significant differences from Kir2.1 T_{50} values ($p < 0.05$); (?) denotes mutants where whole-cell current levels were too small for T_{50} determination.

(C) Correlation of whole-cell current levels and T_{50} values for PIP_2Ab inhibition.

1999), we mutated the conserved basic residues (lysine, Lys; arginine, Arg; and histidine, His) to the uncharged amino acid glutamine (Gln). To assess strength of interaction of channels with PIP_2, we measured the rate of current inhibition by PIP_2 antibody (PIP_2Ab) (Figure 1A) and quantified the kinetics of the effect by measuring the time required for the current to decrease by 50% (T_{50}; Figure 1B). PIP_2Ab competes with the channel for PIP_2 binding, thus reducing current levels. The stronger the PIP_2 interaction with a channel, the slower is the inhibition. We found that the mutant channels H53Q, K182Q, K185Q, R218Q, K219Q, R228Q, and R312Q have significantly faster PIP_2Ab inhibition than the wild-type, suggesting that the interaction with PIP_2 is weakened by each mutation. The size of the whole-cell current for each mutant correlated well with the kinetics of PIP_2Ab-induced inhibition (Figure 1C); strong channel-PIP_2 interactions produced larger currents than weak interactions. This correlation suggests that the decrease in channel-PIP_2 interactions accounted for the decrease in function observed. One exception was K185Q, where the mutation reduced the time required for half maximal current inhibition much more significantly than it did the whole-cell current. Seven of the mutants (R67Q, R82Q, K187Q, K188Q, R189Q, R218Q, and H271Q) produced very little whole-cell current, indicating a crucial importance of those residues for the functional integrity of the channel but preventing T_{50} measurement in five of them (Figure 1B, marked with "?"). Because of the correlation between whole-cell current and channel-PIP_2 interactions, the small current observed with these mutants suggests that these are potentially important sites for maintaining strong PIP_2 interactions with the channel. We used alternative methods (see next two result sections) to assess the importance of these residues.

Polylysine Inhibition Correlates Well with PIP_2Ab Kinetics

To demonstrate whether the Gln mutants we identified in Figure 1 are important for channel-PIP_2 interactions, we also measured rates of current inhibition by poly-Lys. The positively charged poly-Lys, like PIP_2Ab, competes with the channel for the available PIP_2. For Kir2.1, there were two components of inhibition: an almost instantaneous component and a slow component. Only the latter component showed differences among mutants and wild-type channels (Figure 2A). The slow component of poly-Lys current inhibition of Kir2.1 mutants correlated well with the kinetics of PIP_2Ab inhibition (compare Figures 2B and 1C). In addition, we made Cys mutations at the 12 putative PIP_2-interacting residues (Figure 1C, marked with "*" or "?"). Cys mutations tested the importance of these residues for PIP_2 binding by (1) providing another neutral amino acid besides Gln and (2) being able to introduce back a charge of each mutated residue using Cys-modifying reagents (see next section). The Cys mutations of basic residues were made in the background of a control channel (IRK1J), where six native Cys residues were mutated in order to avert irreversible current inhibition after treatment with internal thiol-specific modifying reagents (Lu et al., 1999a). This channel (IRK1J) interacted with PIP_2 in a manner similar to the wild-type channel (Figure 2B). Three of the amino acids that showed very little current when mutated to Gln exhibited enough current upon mutation to Cys to allow reliable assessment of poly-Lys inhibition kinetics (R67, K187, and K188). On the other hand, one of the amino acids that could be assessed when mutated to Gln showed no current when mutated to Cys (K182). Although the PIP_2 interactions with H271Q mutant were not assessed by PIP_2Ab inhibition due to the very small current levels, it was possible to assess this mutant by the faster kinetics of poly-Lys inhibition. Two residues (R82 and R189) still could not be assessed because the Cys mutants did not show detectable membrane current.

Tandem Tetramers with One Single Mutation Allow Investigation of "Dead" Mutants

In order to titrate the functionally lethal effect of the two mutations (R82 and R189), a strategy of concatenating

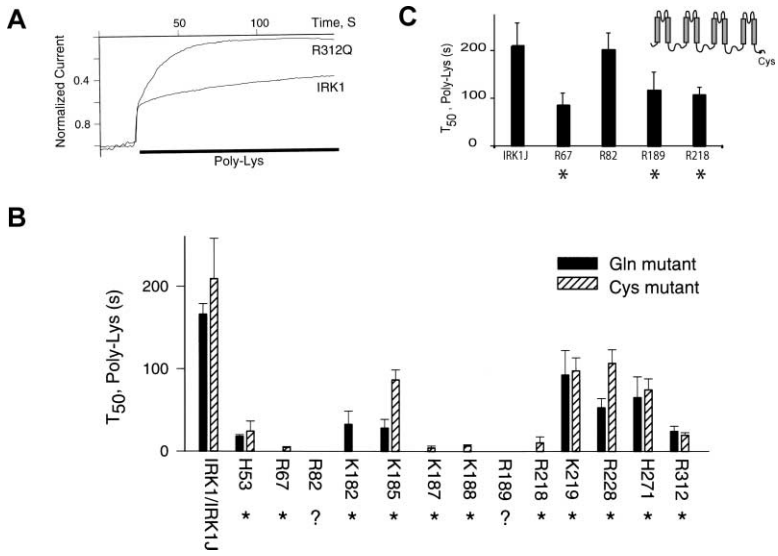

Figure 2. Polylysine Inhibition of Kir2.1 Mutants

(A) Normalized current measured in inside-out macropatches for Kir2.1 wild-type and one of the point mutants studied at −100 mV. There are two components of polylysine inhibition, a very fast PIP_2-independent and a slower PIP_2-dependent component.

(B) Summary data (n = 4–12) of T_{50} values for poly-Lys inhibition for each of the PIP_2-sensitive Gln and Cys mutants tested. The summary data plotted correspond to the half inhibition (T_{50}) of the PIP_2-dependent component. Asterisks denote significant differences from Kir2.1 T_{50} values ($p < 0.05$); (?) denotes mutants with which we were unable to obtain functional expression and therefore determine T_{50} values.

(C) Effect of polylysine on tandem tetramer mutants. Summary data (n = 5–9) of T_{50} values for poly-Lys inhibition for tandem tetramers for each of the mutants tested. Inset: diagram of a tandem tetramer construct, only one of the four subunits bearing the Cys mutation.

four subunits into a tandem tetramer was used (Lu et al., 1999a). We constructed tandem tetramers where three control channel subunits (IRK1J) were linked to one mutant subunit (Figure 2C). The R218C and R67C monomer mutant channels were shown to strongly affect poly-Lys inhibition kinetics (Figure 2) and were also studied for comparison purposes in the tandem tetramer construct bearing a single mutant subunit. For these tetrameric constructs, we were able to see significantly faster kinetics of inhibition compared to control, even with a single mutation present in the channel. For R189, the mutation in the tetramer made the inhibition significantly faster than control, but the R82 mutation did not have an effect in channel-PIP_2 interactions, suggesting a PIP_2-independent control on channel activity by this mutation. This result cautions that small whole-cell currents do not necessarily imply weaker channel-PIP_2 interactions.

In summary, we identified 12 residues that changed channel-PIP_2 interactions when neutralized. Two residues were found in the N terminus (H53 and R67), 5 clustered in the C-terminal region proximal to the membrane (K182, K185, K187, K188, R189), 3 clustered in a distinct C-terminal region ~40 residues away from the membrane (R218, K219, R228), and 2 scattered in the distal C terminus (H271 and R312).

Application of MTSEA Strengthens PIP_2-Channel Interaction

By systematically replacing the positively charged residues with the neutral Cys residue, we can chemically restore a positive charge to the mutant through the use of water-soluble sulfhydryl-specific reagents. Methanethiosulfonate ethylammonium (MTSEA) forms a covalent bond with accessible sulfhydryl groups present in the Cys and has a similar side chain length and the same NH_3^+ side chain as Lys. In order to use such sulfhydryl-modifying reagents, we used as control the mutant channel IRK1J (Lu et al., 1999a), which has no intracellular reactive Cys. Charged residues were then mutated to Cys on the background of this channel. Most of the 12 identified mutants showed significantly stronger PIP_2 interaction after MTSEA was covalently bound to the channel (Figure 3). This effect seemed more pronounced on the Lys residue mutants, consistent with the fact that a Cys covalently bound to MTSEA is more similar to a Lys than to an Arg residue. A similar but less dramatic effect was observed when the larger methanthiosulfonate ethyltrimethylammonium (MTSET) was used instead of MTSEA (see H53C mutant in Figure 3C). Moreover, an inhibition of the current was observed after the negatively charged methanethiosulfonate sulphonatoethyl (MTSES) was applied to the H53C mutant channel. Poly-Lys inhibition after MTSES application was "instantaneous" (<1 s), suggesting weaker interaction with PIP_2 when a negative charge is present at the 53 position (data not shown). This result suggests that not only charge but also the characteristics and positioning of the charged group are important for interactions with PIP_2. The presence of different charged head-groups, either NH_3^+ (MTSEA) or $N(CH_3)_3^+$ (MTSET), was likely to affect their binding to the phosphate head-groups of PIP_2. Furthermore, a very dramatic increase in the current level was seen when MTSEA reacted with H53C, R67C, K187C, K188C, R218C, and K219C (e.g., Figures 3A and 5A). A similar increase in current had been previously observed for the K219 residue (Lu et al., 1999b), but the reasons for the effect were not clear. These results suggest that the decrease in current levels observed for the PIP_2 mutants can be reversed once the positive charge is replaced and the PIP_2 binding site is reestablished. Moreover, the results with MTSEA support the hypothesis that mutations in positively charged residues influence PIP_2 interactions directly and not allosterically. Repetitive applications of poly-Lys without MTSEA treatment did not change the kinetics of inhibition (data not shown).

There were three residues (R67, R312, and H271), which when neutralized caused significant weakening of the PIP_2-channel strength of interactions but showed only mild (R67/R312) or no significant (H271) strengthening for interactions with PIP_2 by MTSEA application. In order to assess whether this was due to either the

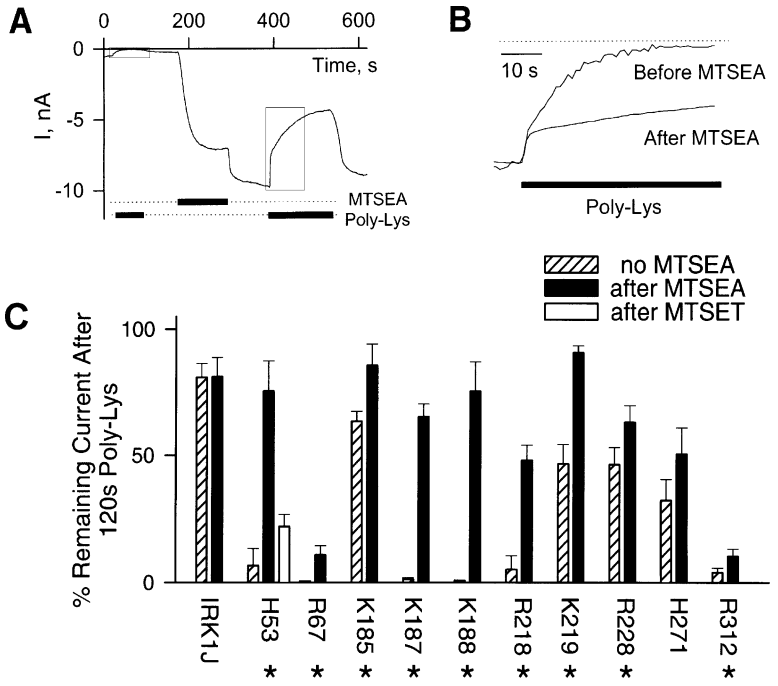

Figure 3. Effect of MTSEA Application on the Kinetics of Polylysine Inhibition

(A) Current measured from an inside-out macropatch for the H53C mutant at −100 mV. Note the inhibition before exposure to MTSEA, the increase in current levels during MTSEA application, the inhibition by poly-Lys after MTSEA was covalently bound to the channel, and the recovery of the current after a wash of poly-Lys following a wash of MTSEA. Current levels increased even further after a wash of MTSEA. Highlighted areas expanded in (B).

(B) The kinetics of current inhibition by poly-Lys before and after MTSEA are compared. Current is scaled for comparison purposes.

(C) Summary data (n = 3–9) of percentage of current remaining after a 120 s of exposure to poly-Lys before and after a 100 s MTSEA application. Asterisks denote significant differences from T_{50} before MTSEA values ($p < 0.01$).

inaccessibility of these residues to MTSEA or to the requirement of a particular residue at that position, we mutated these sites to other positive charged residues (R67K, R312K, H271K, H271R). In the cases of R67K and R312K, the alternative positively charged residues increased PIP_2-channel interaction, but the interaction was weaker than for the wild-type channel. T_{50} for poly-Lys inhibition was 25 ± 5 s (R67K, n = 4) and 53 ± 11 s (R312K, n = 5) when compared to 4.7 ± 0.9 s (R67C, n = 4) and 20 ± 3 s (R312C, n = 11) for neutral amino acids mutations and 209 ± 49 s for wild-type channels. For the H271 mutants, both mutations to other basic residues were nonfunctional, suggesting that factors other than the charge of the residue were important for the weakening effect of the neutralization mutations at this position and implying that this residue does not share electrostatic interactions with PIP_2. Mutations at the H271 residue had a more dramatic effect in whole-cell current (Figure 1B) than in channel-PIP_2 interactions (Figure 2B), suggesting that the effect of this mutation is not fully accounted for by the observed decrease in channel-PIP_2 interactions. The K182C mutant was nonfunctional both before and after MTSEA application. To assess the importance of the charge at this position, we mutated the residue to another positively charged amino acid (K182R). K182R was also nonfunctional, suggesting that the charge per se of this residue was not important for maintaining these interactions. These results imply that both H271 and K182 do not share electrostatic interactions with PIP_2.

Equivalent Residues in Kir1.1 Also Affect Channel-PIP_2 Interactions

Because we use Kir2.1 as a model to test basic amino acids that were conserved among Kir channels (Figure 4A), we proceeded to evaluate whether the residues that affected PIP_2-Kir2.1 interactions served the same role in other channels, such as Kir1.1. We tested the effects of corresponding neutralizing mutations in Kir1.1. The residues in Kir1.1 corresponding to Kir2.1(R67) and Kir2.1(R228) are not positively charged and thus were not tested. Similarly, the equivalent residue to Kir2.1(R189) (R188 in Kir1.1) has already been shown to affect Kir1.1-PIP_2 interactions (Huang et al., 1998), and it was not tested further. We found that mutations of 7 of the 9 residues tested in Kir1.1 had significantly weaker inhibition kinetics when compared to control (Figure 4B). Neutralizing mutations in Kir1.1 residues that correspond to the Kir2.1-PIP_2 interacting residue K188 did not affect Kir1.1-PIP_2 interactions. In Kir2.1, K188 mutations showed large weakening effects on channel-PIP_2 interactions (Figure 2). Because of this difference between Kir2.1 and Kir1.1 channels in PIP_2 interactions, we tested another candidate neighboring residue in Kir1.1 for possible interactions with PIP_2. The equivalent residue to Kir2.1(E191) is a Lys in Kir1.1. This basic residue is unique to the Kir1.1 channel at this position, and it also affected Kir1.1-PIP_2 interactions when mutated to Glu (for poly-Lys inhibition: T_{50} = 18 ± 7 s [n = 3] and 174 ± 27 s [n = 11] for the Glu mutant and wild-type channels, respectively). These results indicate that although neutralization of the Kir1.1 residue equivalent to the Kir2.1 residue K188 showed no significant effects on channel-PIP2 interactions, a neighboring basic residue served a similar role in Kir1.1 interactions with PIP_2 as residue K188 does in Kir2.1. Moreover, this result suggests that although conserved charges affect channel-PIP_2 interactions for both channels, variations in the position of these charges within the specific PIP_2-interacting channels are possible. Consistent with this result, differences for the specificity for phosphoinositide activation have

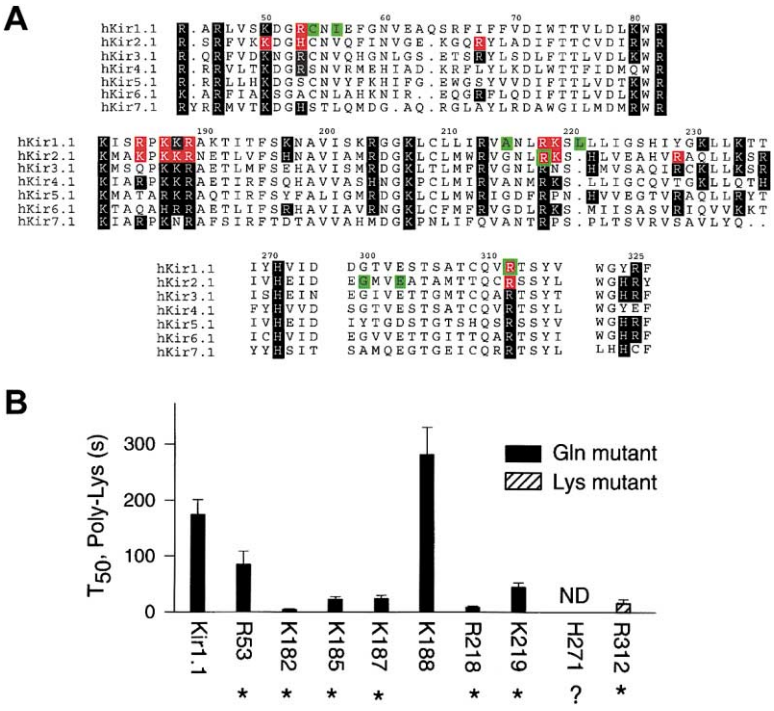

Figure 4. Mutations in Conserved Kir-Channel Residues Affect Both Kir2.1 and Kir1.1

(A) Alignment of N and C termini regions of Kir channels. Residues marked in black are basic amino acids highly conserved among the Kir families. All of these conserved basic residues were mutated to neutral amino acids and were tested in Kir2.1 for effects on interactions with PIP_2. Residues marked in red were conserved basic amino acids found to affect channel-PIP_2 interactions when neutralized. Residues marked in green are associated with naturally occurring mutations that affect channel-PIP_2 interactions. Residue numbers refer to positions in Kir2.1.

(B) Mutants in Kir1.1 corresponding to those identified in Kir2.1 also affect PIP_2-channel interactions. Summary data ($n = 4$–8) of T_{50} values for polylysine inhibition for each of the Gln and Lys mutants tested. Numbers refer to the corresponding residue in Kir2.1. Asterisks denote significant differences from wild-type Kir1.1 T_{50} values ($p < 0.005$); (?) denotes mutants where whole-cell current levels were zero or too small for T_{50} determination.

been found for different Kir channels (Rohacs et al., 2002, Biophys. J., abstract; T.R., C.M.B.L., T.J., D.E.L., unpublished data). Kir2.1 has been shown to be specific to $PI(4,5)P_2$ when compared to $PI(3,5)P_2$, $PI(3,4)P_2$, and $PI(3,4,5)P_3$ (Rohacs et al., 1999).

We could not measure any currents from either the H271Q or the R312Q equivalent Kir1.1 mutants. Consistent with our findings, the Kir1.1 channel lacks activity when the equivalent residue to Kir2.1(H271) is mutated either to a neutral or to a positively charged amino acid (Chanchevalap et al., 2001). When we mutated the residue equivalent to R312 to a Lys, we found that the mutated channel had weaker interaction with PIP_2 than the wild-type channel, a similar effect to that obtained by the corresponding mutation in Kir2.1 (Figure 5A). This result suggests that the charge at this position is not the only important factor for channel-PIP_2 interactions (see next section).

In summary, the majority of PIP_2-interacting Kir2.1 charges fulfill a similar role in Kir1.1. From the nine conserved charges between the two channels, only one (position 188) behaved differently. The interacting residue K188 in Kir2.1 was represented by the Lys residue present in Kir1.1 in the equivalent position to Kir2.1(E191). Thus, a similar design for conserved charges to interact with PIP_2 is seen in both the Kir2.1 and Kir1.1 channels.

Channel-PIP_2 Interacting Sites Are Associated with Channelopathies

Kir2.1-neutralizing mutations at R218 (R218Q/W) have been associated with Andersen's syndrome (Plaster et al., 2001), and the Kir1.1 mutations at R311 (R311Q/W) (equivalent to R312 in Kir2.1) are two of the genetic defects associated with the antenatal variant of Bartter's syndrome (Schulte et al., 1999). We identified these residues as conserved PIP_2 interaction sites (Figures 1, 3, and 4). The decrease in whole-cell current observed for these mutants correlated well with the faster kinetics of PIP_2Ab inhibition (Figure 1C), suggesting that decrease in channel-PIP_2 interactions caused loss of function. In order to gain insight on the molecular mechanisms underlying these diseases, we studied in greater detail the charge dependency of PIP_2 interactions for these sites. For Kir2.1 and Kir1.1, there is a large decrease in PIP_2 interactions when these residues are mutated to neutral amino acids (Figure 5A). For both channels and sites, the Lys mutant had a stronger interaction with PIP_2 than that of the neutral mutant, suggesting the existence of electrostatic interactions of these sites with PIP_2 (Figure 5A). A similar strengthening of channel-PIP_2 interaction was observed for the Kir2.1(R218C/R312C) mutants when MTSEA was applied (see Figure 3C). In addition, for Kir2.1(R218C), when MTSEA was applied, reestablishing the residue charge, a large increase in current was observed (Figure 5B). The Kir1.1(R311Q/W) mutants did not have enough current to be tested in inside-out patches, while the Kir1.1(R311K) mutant displayed large current levels, which allowed characterization. In addition, Kir1.1(R311K) showed weaker interaction with PIP_2 than the wild-type channel, showing that mutations on this residue were indeed affecting channel-PIP_2 interactions as in the case of the Kir2.1 channel. These results suggest that similarly to the Kir2.1(R312) residue, the charge of the Kir1.1(R311) residue was important for PIP_2 interactions. Although a positively charged residue at both sites in the two channels showed stronger interaction than a neutral amino acid, properties of Arg residues other than charge were also important to maintain the strongest interaction with PIP_2.

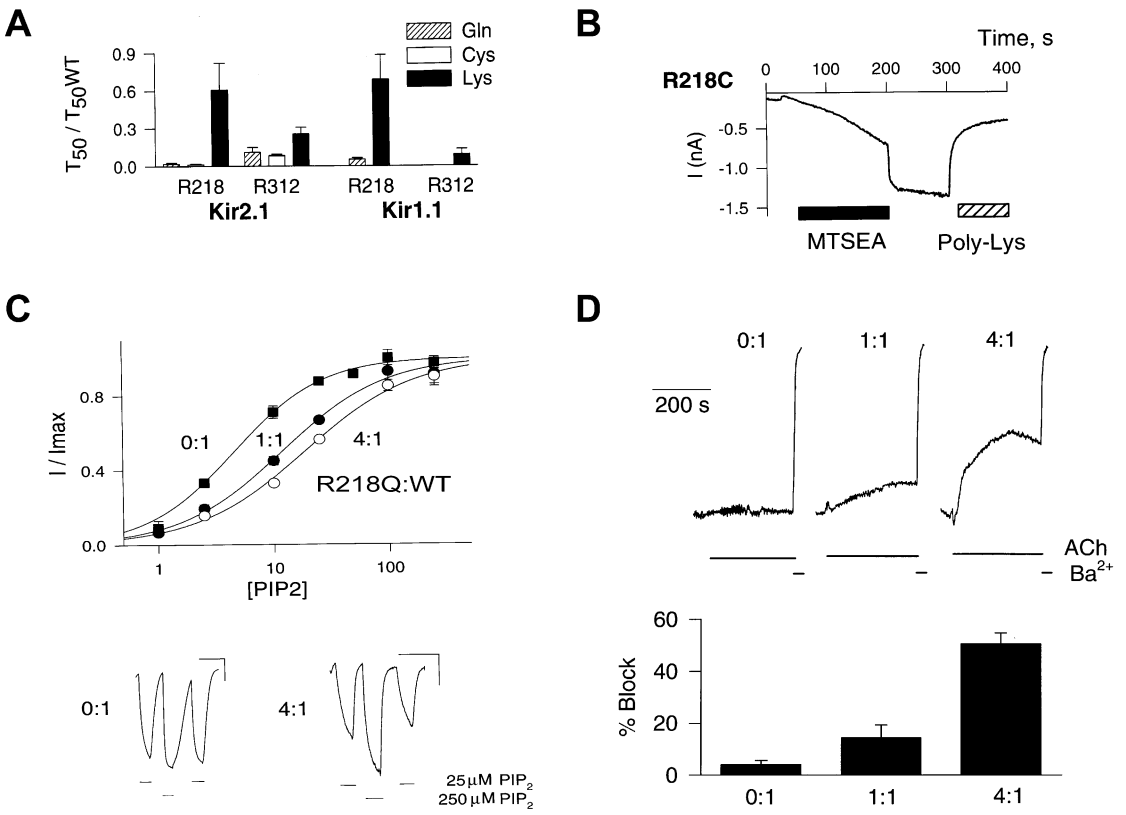

Figure 5. Mutation in Channel-PIP_2 Interacting Residues Affects PIP_2 Affinity and Channel Regulation

(A) Charge of R218 and R312 residues determines the strength of channel-PIP_2 interactions and current levels. Summary data (n = 3–9) of T_{50} values for polylysine inhibition for each of the channel mutants tested. Numbers refer to the corresponding residue in Kir2.1.

(B) Current measured from an inside-out macropatch for the R218C mutant at −100 mV. Note that R218C channel activity increases after MTSEA is bound to the channel.

(C) Coexpression of mutant subunits decreases PIP_2 affinity with the channel. Bottom: representative current trace measured in inside-out macropaches in response to DiC_8PIP_2 for wild-type expressed alone (0:1) and R218Q and wild-type combinations (1:1 and 4:1 ratios). Scale bars represent 2 nA and 200 s (0:1) and 1 nA and 200 s (4:1). Top: summary data for Kir2.1 subunits expressed alone and together with R218Q. Each data point is an average of two to five experiments. Data are expressed as a percentage of the response induced by 25 μM DiC_8PIP_2. Solid line is a Hill fit to all data points.

(D) Mutation associated with Andersen's syndrome confers sensitivity to PIP_2 hydrolysis occurring via M1 receptor stimulation. Top: whole-cell current measured in *Xenopus* oocytes expressing either Kir2.1 or Kir2.1/R218Q channels (4:1 ratio). 10 μM ACh is applied to the extracellular solution. Bottom: summary data of inhibition of various mutant:wild-type combinations elicited by ACh application.

Expression of Mutant Subunits Affects Channel-PIP_2 Affinity

Andersen's syndrome occurs either sporadically or as an autosomal dominant trait, where both mutant and wild-type subunits are expressed in the patient. We have coexpressed Kir2.1 and the R218Q mutant channel in *Xenopus* oocytes at a 1:1 and a 4:1 mutant to wild-type subunit ratio. We examined the sensitivity of these channels to PIP_2 by applying to inside-out macropatches short chain water-soluble PIP_2 (DiC_8) at different concentrations (Rohacs et al., 2002). The dose response of the Kir2.1/R218Q channels to PIP_2 is right-shifted (Figure 5C) when compared to wild-type subunits expressed alone. A typical trace of application of 25 and 250 μM DiC_8-PIP_2 to a patch containing either wild-type channel or channels containing mutant subunits is shown on the bottom panel of Figure 5C. A concentration of 25 μM PIP_2 gives close to maximum activation for wild-type channels but only activates about half of the maximum current for channels containing mutant subunits. These results show that mutations in R218 decrease PIP_2 affinity. The estimated EC_{50} for each curve is 4.6 ± 0.7 μM for wild-type channels (0:1 ratio), 12 ± 2 μM for the coinjection at a 1:1 ratio, and 19 ± 3 μM for the coinjection at a 4:1 mutant to wild-type subunit ratio. The Hill coefficient for each curve was 1.2 ± 0.2 for wild-type and 1.0 ± 0.1 and 0.9 ± 0.1 for 1:1 and 4:1 ratio of coinjection with mutant subunits, respectively, not precluding that only one PIP_2 molecule binds to the channel. If more than one PIP_2 molecules binds to the channel, then Figure 5C suggests that either PIP_2 binding sites are independent or the cooperativity is low.

Expression of Disease Subunits Confers Sensitivity to Agonist-Induced PIP_2 Hydrolysis

The muscarinic (M1) receptor is known to couple to phospholipase C (PLC) through Gq. The substrate for PLC is PIP_2. When M1 receptors and Kir channels are coexpressed in *Xenopus* oocytes and ACh is applied, PIP_2 is hydrolyzed, and Kir currents can be inhibited

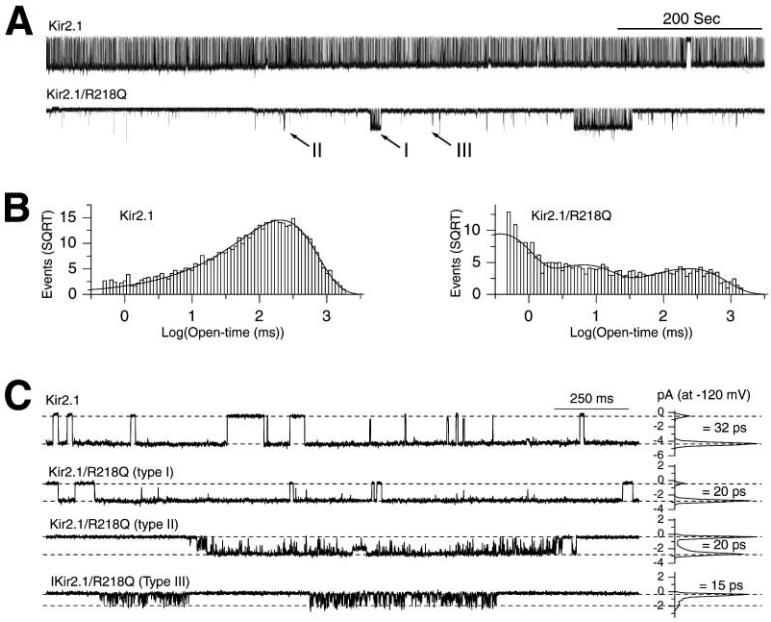

Figure 6. Expression of a Disease Mutant Subunit Affects Single-Channel Kinetics

(A) Single-channel recordings of Kir2.1 expressed alone and with R218Q (20:1 ratio). Representative single-channel traces recorded in cell-attached mode from *Xenopus* oocytes for Kir2.1 and wild-type (WT) and R218Q mutant subunits coexpressed (WT/R218Q) at −120 mV.

(B) Open-time histogram of traces shown in (A). Traces could be fit with either one exponential component for wild-type channels expressed alone and three for WT/R218Q.

(C) Left: representative traces at an expanded time scale (as indicated with arrows in [A]). Right: all points amplitude histogram for the traces shown in the left panel.

(Kobrinsky et al., 2000). The extent of current inhibition observed is dependent on the strength of channel-PIP$_2$ interactions (H. Zhang et al., 2002, Biophys. J., abstract; H.Z., C.M.B.L., D.E.L., T. Mirshahi, unpublished data). No inhibition is observed for the strongly interacting Kir2.1 (Figure 5D). From the study of the kinetics of poly-Lys block, we established that mutations associated with Bartter's and Andersen's syndromes decrease the strength of PIP$_2$ interaction with the channel. Coexpression of wild-type and mutant subunits decreased but did not abolish current. We coexpressed the Andersen's-associated mutant R218Q subunits and wild-type Kir2.1 subunits (in ratios 1:1 and 4:1, R218Q:WT) with M1 receptors. Channel inhibition upon ACh activation of M1 receptors was seen for channels where mutant subunits were coexpressed and not for wild-type channels. The extent of the inhibition was dependent on the number of mutant subunits expressed, suggesting it depended on the number of mutant subunits present in the channel (Figure 5D).

Expression of Mutant Subunits Strongly Affects Channel Gating

We have expressed Kir2.1 and R218Q subunits in *Xenopus* oocytes and examined their unitary currents on cell-attached patches. When Kir2.1 subunits were expressed alone, we observed channels with high open probability (P_0), long mean open time, brief closures, and long burst duration (Figure 6A). The open-time kinetics of Kir2.1 could be described reasonably well with a one-component exponential function. The open-time constant from two recordings containing only one channel was 190 ± 5 ms, consistent with previous reports (e.g., Choe et al., 1999). No channel activity was observed in patches where R218Q subunits were expressed alone. For oocytes where Kir2.1 and R218Q subunits were coexpressed (ratio 1:20), single-channel recordings suggest that Kir2.1 wild-type subunits associate with R218Q subunits to form functional channels with dramatically different gating properties than wild-type channels. Due to the low open probability of these channels, we could not assess the number of channels in each patch. Even though there seemed to be multiple channels present in each patch (rare simultaneous openings to multiple levels), most of the openings observed were unitary. The open-time kinetics of four separate recordings from Kir2.1 coexpressed with R218Q subunits can be fitted with three-component exponential functions (Figure 6B). The three open-time constants for Kir2.1/R218Q are 0.53 ± 0.08, 6.4 ± 0.6, and 195 ± 30 ms, respectively. The longest component of the open-time kinetics of Kir2.1/R218Q is similar to the single open-time constant of the wild-type channels. These components seem to correspond to three distinct populations displayed in the recordings, type I, type II, and type III, as indicated with arrows in Figure 6A and displayed in the expanded time scale in Figure 6C. Rare double openings of different populations were observed, suggesting that these distinct kinetic channel behaviors were due to different channel populations in the patch. Type I displays almost identical intraburst single-channel kinetics as wild-type Kir2.1 (Figure 6C). For the representative record shown, the mean open time was 170 ms and open probability was 0.84. Nevertheless, type I has a much shorter burst duration, as can be visually seen in Figure 6. There is also a 30% decrease in single-channel conductance observed for this population when compared to wild-type channels (18 ± 1 pS for Kir2.1/R218Q and 32 ± 1 pS for Kir2.1 alone). Although the effect on the conductance is less dramatic than the change in gating observed, it suggests that coexpression of mutant subunits also affects permeation. Type II displays even shorter burst duration than type I, and its intraburst open time is much shorter than either type I or wild-type Kir2.1. For the representative record shown in Figure 6C, the mean open time is 7.5 ms. Nevertheless, the open probability within a burst is comparable to Kir2.1 expressed alone (0.79 for the record shown). Type III (mean open

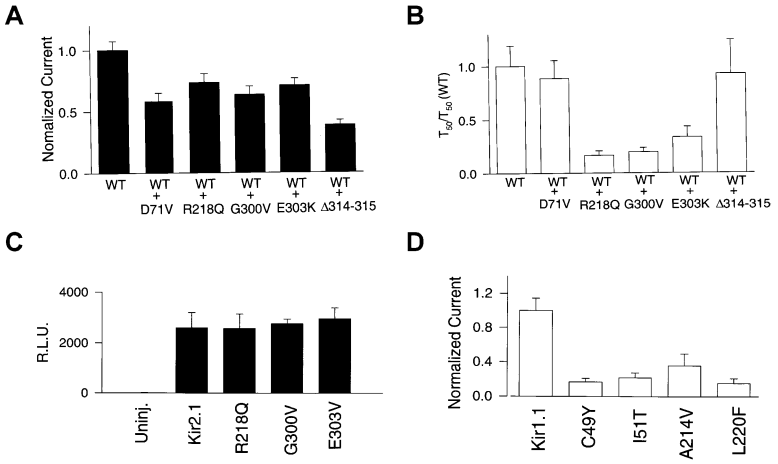

Figure 7. Several Disease Mutants Affect Channel-PIP_2 Interactions

(A) Summary data (n = 10–12) of whole-cell current levels for each of the Kir2.1 mutants coexpressed with wild-type Kir2.1 at a ratio of 1:1.

(B) Summary data (n = 5–9) of normalized T_{50} values for poly-Lys inhibition for each of the Kir2.1 mutants coexpressed with wild-type Kir2.1 at a ratio of 1:1.

(C) Surface expression of Kir2.1 and each of the mutants tested expressed alone and uninjected oocytes. Surface expression was performed on single oocytes injected with 5 ng RNA using anti-HA antibody (n = 11). R.L.U., relative light units.

(D) Summary data (n = 4–5) of normalized T_{50} values for poly-Lys inhibition for each of the Kir1.1 mutants tested.

time of 0.56 ms) displays a much lower open probability within a burst (0.12 for the representative record shown). It is compelling to suggest that the three types of single-channel behaviors observed correspond to different populations of channels displaying different mutant/wild-type stoichiometries. These would be: type I, containing one mutant and three wild-type subunits; type II, with two mutant and two wild-type subunits; and type III, with one wild-type and three mutant subunits. This assertion, however, remains to be rigorously proven. Channels formed with four mutant subunits have not been observed at the single-channel level. Because Andersen's syndrome is an autosomal dominant disease, both wild-type and mutant subunits are made in the cell, and a population of channels with different stoichiometric combinations of wild-type and mutant subunits is likely to be formed and expressed in patients.

Several Disease Mutations Not Involving Basic Residues Decrease Channel-PIP_2 Interactions

There are mutations in eight amino acids in Kir2.1 suggested to cause the developmental and episodic electrical phenotypes of Andersen's syndrome (Plaster et al., 2001). Six of these mutations occur in the intracellular portion of the channel (D71V, R218Q/W, G300V, E303K, Δ314-315). Since Andersen's syndrome occurs either sporadically or as an autosomal dominant trait, both mutant and wild-type subunits are expected to be expressed in the patients. Two of the mutations (D71V and R218W) were shown to decrease current levels when coexpressed with wild-type subunits and showed no current when expressed alone (Plaster et al., 2001). We coexpressed wild-type with mutant subunits for mutations occurring in each of the residues in the intracellular domains of Kir2.1 (D71V, R218Q, G300V, E303K, Δ314-315). Current levels were significantly decreased for wild-type channels (2 ng/oocytes) when coexpressed with each of the mutant subunits (2 ng wild-type and 2 ng mutant), showing that in all cases, the mutant subunit was coassembling with the wild-type subunit (Figure 7A). Current levels were reduced even though twice the total amount of RNA was injected in oocytes where wild-type and mutant subunits were coexpressed. No current was observed when the mutant subunits were expressed alone (data not shown). For three of the five channels formed by mutants (R218Q, G300V, E303K) and wild-type subunits, the T_{50} for poly-Lys inhibition was significantly faster than for wild-type channels (Figure 7B). We explored the possibility that decreasing trafficking of the protein to the cell surface might contribute to the decreased functional expression observed in disease mutants. We measured the surface expression of wild-type Kir2.1 and each of the mutants that decreased channel-PIP_2 interactions using single oocytes and a fluorimetric assay (Zerangue et al., 1999). Disease-associated mutations did not significantly change the channel surface expression to the plasma membrane (Figure 7C), even though mutations abolished channel function. For two mutations (D71V and Δ314-315), there was no decrease in the strength of PIP_2 interactions. Either these mutations do not affect PIP_2 interactions with the channel or these are dominant-negative mutations, where only channels formed by wild-type subunits alone displayed current.

We also tested five additional Kir1.1 mutations of residues that are not positively charged and are associated with Bartter's syndrome: C49Y, I51T, D74Y, A214V, L220F (International Collaborative Study Group for Bartter-like Syndromes, 1997; Schulte et al., 1999; Schwalbe et al., 1998). Bartter's syndrome is an autosomal recessive disorder. Expressing mutant channels alone simulates the effect of the disease. One of the mutant channels (D74Y) did not show current when expressed alone. The other four mutant channels (C49Y, I51T, A214V, and L220F) had significantly faster inhibition by poly-Lys, suggesting weaker PIP_2 interactions with these channels (Figure 7C). Our results suggest that a decrease in channel-PIP_2 interactions underlies the molecular mechanism of Andersen's and Bartter's syndromes when these mutations are present in patients.

Discussion

All Kir channels tested thus far have been shown to interact with PIP_2. These interactions are crucial for channel activity. In general, PIP_2 binds to electrostatically polarized domains in proteins (Kavran et al., 1998). Binding occurs through electrostatic interactions and shows varying levels of head-group specificity (Ferguson et al., 1995). Therefore, basic amino acids present

in intracellular regions of the channels are putative PIP_2 interacting sites. A few conserved positively charged residues have been shown to either decrease channel activity or affect channel-PIP_2 interactions when neutralized (equivalent residues to the Kir2.1 residues: K188 [Kir6.2] [Baukrowitz et al., 1998], R189 [Kir6.2] [Baukrowitz et al., 1998] [Kir1.1] [Huang et al., 1998], R218 [Kir2.1] [Zhang et al., 1999], R228 [Kir2.1] [Zhang et al., 1999], and R312 [K_{ATP}] [Shyng et al., 2000]), but only one of those was implicated as a PIP_2 binding site (R189 [Kir1.1] [Huang et al., 1998]). In this study, we aimed to identify conserved PIP_2 interaction sites with Kir channels. Our systematic and comprehensive approach yielded ten Kir2.1 basic residues that interact with PIP_2. All but one of the corresponding residues in Kir1.1 show similar interactions with PIP_2. Mutations in both Kir2.1 and the related Kir1.1 channels lead to disease. Expression of subunits bearing mutations in a PIP_2 interaction site associated with Andersen's syndrome mutations caused changes in PIP_2 affinity and a decrease in channel open probability. Our study shows that decrease in channel function due to weakening of channel-PIP_2 interactions is linked to channelopathies.

Identification of Both C- and N-Terminal Residues of Kir Channels that Interact with PIP_2

There are 45 basic amino acids (Lys, Arg, or His) in the cytoplasmic domains of Kir2.1. Twenty-seven of these basic residues are highly conserved among the Kir channels (present in at least either half of the subfamilies or half of the family members). Neutralization of 12 basic amino acids at both N and C termini affects channel-PIP_2 interactions. With the use of Cys-modifying reagents and mutation to charged and neutral residues, we have shown that the charge per se of 10 of the 12 residues is important for Kir2.1-PIP_2 interactions (marked in red in Figure 4A), suggesting that these 10 residues are part of one or more PIP_2 binding sites. In addition to rigorously testing interactions of all conserved C-terminal basic residues in Kir channels with PIP_2 and identifying eight interacting sites (K185, K187, K188, R189, R218, K219, R228, and R312), the present study identifies important N-terminal PIP_2 interactions, as observed for H53 and R67. On the other hand, residues where mutations to both positively charged and neutral amino acids had similar effects (i.e., H271 and R182) do not seem to interact with PIP_2. Charge neutralization of these two amino acids was not responsible for the decrease in current levels and the weakening of the PIP_2-channel interactions observed. Thus, an allosteric mechanism and possibly conformational changes that affect interactions with PIP_2 at different sites are likely to be involved. These two residues that allosterically affect Kir2.1-PIP_2 interactions (K182 and H271) are both present and cause a similar effect in Kir1.1, suggesting that the structural consequences of these mutations are conserved among these channels. There are 10 Kir2.1 residues that form one or more PIP_2 binding sites. Eight of these amino acids are conserved in Kir1.1. Seven of these residues affect Kir1.1-PIP_2 interactions when neutralized in a similar fashion as seen with Kir2.1, suggesting that interactions with PIP_2 are conserved among these channels.

PIP_2 possesses two negative phosphate headgroups. The structure of a number of protein domains that interact with PIP_2 has been determined, and the residues that interact with PIP_2 have been either identified (e.g., Ferguson et al., 1995; Ford et al., 2001) or proposed (Itoh et al., 2001; Mao et al., 2001). These are, among others: the PH domain of PLCδ, the epsin NH_2-terminal homology (ENTH) domain, and a Lys-rich domain in the adaptor protein 180 (AP180). For these domains, a cluster of basic residues marks the location of the phosphoinositide binding site. The number of basic residues involved in the formation of the binding pocket varies among phosphoinositide binding domains. Five basic amino acids are present in the AP180 protein, 7 in the PH domain of PLCδ, and 9 in the ENTH domain. Some of the residues that form the binding pocket do not share direct contact interactions with the phosphate head groups. The phosphoinositide binding site of the ENTH domain was identified by a combination of a comprehensive analysis of all amino acids in the protein with nuclear magnetic resonance and mutagenesis (Itoh et al., 2001). The severity of the effect on PIP_2 binding of mutations varied among the 6 basic amino acids tested. It was proposed that residues that had a more dramatic effect on binding would be forming salt bridges with the phosphate groups. Of the 10 amino acids that we found to form the PIP_2 binding pocket, H53, R67, K187, K188, K189, R218, and R312 have a more dramatic effect, suggesting that these residues might share direct contact interactions with the phosphate groups. Other residues (K185, K219 and R228) might form a binding pocket but not directly touch the PIP_2 molecule. The relatively large number of amino acids found to be important in PIP_2 interactions with the channel might reflect more than one binding site for PIP_2. However, the number of residues found is not inconsistent with a single binding site. There is evidence that C and N termini of Kir channels interact with each other (Qu et al., 2000; Schulte et al., 1999). It will be important, once specific N- and C-terminal interaction sites are mapped, to compare their spatial localization to the PIP_2-interacting basic residues identified in our study.

Our results do not exclude the possibility that electrostatic intraprotein interactions are reestablished with the application of positively charged Cys-modifying reagents and that allosteric changes in protein conformation affect the channel-PIP_2 interactions, although this possibility appears unlikely. Our reasoning for this interpretation stems from the observation that the residues that MTSEA modifies to strengthen channel interaction with PIP_2 are water accessible, which argues against intraprotein interactions. Moreover, these mutated channels before and after modification show similar functional properties (i.e., have the same kinetics of activation and inward rectification properties as wild-type channels). Thus, intraprotein allosteric effects are rather unlikely, as they would have to be large enough to expose to the hydrophilic environment previously buried residues without affecting basic channel properties.

Two of the Identified PIP_2 Interaction Sites Are Important for Channel Regulation by PKA

Several Kir channels have been shown to be sensitive to PKA phosphorylation, including Kir1.1 (Xu et al., 1996) and Kir2.1 (Dart and Leyland, 2001). Two of the residues

we identified as PIP_2 interacting, R218 and R312 for Kir2.1, are part of two putative PKA phosphorylation consensus motifs (RXS, where X is polar) (Kennelly and Krebs, 1991) conserved in all Kir channels. Both these motifs have been shown to be phosphorylated in Kir1.1 (Xu et al., 1996). We showed that when the charge of these residues is maintained, the interaction with PIP_2 is preserved (Figures 3C and 5A). Yet, all mutations that destroy the phosphorylation motif (R217/R218K/Q and R311/R312K/Q) weakened chanel-PIP_2 interactions, suggesting complex interactions at these sites. Consistent with our results, mutations of both Ser residues (S219A/S219D and S313A/S319D) have been shown to decrease Kir1.1-PIP_2 interactions (Liou et al., 1999). These results may reflect: (1) a decrease in channel-PIP_2 interaction caused by lack of phosphorylation on the particular site, (2) a simple decrease in PIP_2 binding by disruption of the existing interactions by the mutations, or (3) a combination of both effects (1 and 2).

PKA modulation of Kir1.1 has been shown to increase Kir1.1-PIP_2 interactions (Liou et al., 1999). We attempted to directly test PKA regulatory effects for the wild-type and mutant channels, with and without coexpression of A kinase anchoring protein 79 (AKAP79). Unfortunately, we were not able to reproduce the reported increase in whole-cell currents when PKA was activated in *X. laevis* oocytes (Ali et al., 1998). This might have been due to the high and possibly variable levels of cAMP in oocytes (Perets et al., 1996). Excised Kir1.1 patches showed loss of activity over time that could not be restored by either PKA phosphorylation or PIP_2 application, preventing the measurement of effects of PKA phosphorylation to channel sensitivity to PIP_2 levels. Further experiments in different expression systems may be needed to clarify this point. Although our results suggest that PKA phosphorylation might act by modulating PIP_2 binding at R217/R311 residues, details underlying this effect need to be further clarified in future studies.

Channel-PIP_2 Interactions as a Novel Mechanism for Channelopathies

PIP_2-interacting residues are associated with channelopathies. Kir2.1 mutations in R218 (R218Q/W) have been associated with Andersen's syndrome, a disease characterized by periodic paralysis, cardiac arrhythmias, and dysmorphic features (Plaster et al., 2001). Kir1.1 mutations in R312 (R312Q/W) are one of the genetic defects associated with the antenatal variant of Bartter's syndrome, a disease characterized by polyhydramnios, premature delivery, hypokalemic alkalosis, and hypercalciuria (Schulte et al., 1999). Mutations in both sites decrease channel-PIP_2 interactions. We showed that generally, and in particular for R218Q and R312Q, neutralizing mutations that affect channel-PIP_2 interactions affect whole-cell current and strength of interaction in a similar way. This correlation suggests that the weakening of PIP_2 interactions causes the observed decrease in function. We showed that coexpression of wild-type and R218Q mutant subunits shifts the affinity of the Kir2.1 channel to PIP_2. This implies that at a similar level of membrane PIP_2, a channel containing mutant subunits would display lower activity. At the single-channel level, channels bearing mutant subunits show a dramatic decrease in channel open probability. Generally, studies agree that higher PIP_2 levels in the membrane increase Kir channel function by decreasing channel mean closed time (Enkvetchakul et al., 2000; Rohacs et al., 2002). Similarly, an increase in mean closed time, as we observed, would be expected for the mutations that decrease PIP_2 interactions with Kir channels. Thus, a decrease in channel-PIP_2 interactions and consequent decrease in activity can account for the disease phenotype. Variation in the expression of alleles might be responsible for changes in the severity of the disease in the case of Andersen's syndrome, where both mutant and wild-type subunits are present.

Our results show that coexpression of wild-type and mutant subunits decreases but does not abolish channel activity. Mutant and wild-type subunits form channels that express current and have a weaker interaction with PIP_2. Kir2.1 contributes to cell excitability and resting membrane potential in excitable tissues including heart, brain, and skeletal muscle. Cell excitability in these tissues is under constant regulation. For instance, heart rate and contractility are continually altered in response to changing cardiovascular demands and stresses by means of autonomic stimulation. Changes in autonomic stimulation of the heart appear to be linked to the precipitation of both hereditary and acquired ventricular arrhythmias. Autonomic receptors (muscarinic M1 and α-adrenergic α1A) and angiotensin (AT1) receptors, among others, can couple to G proteins of the Gq/G11 family and, when activated, stimulate PLC. The substrate for PLC is PIP_2; thus, agonist stimulation of the receptor causes reduction of PIP_2 in the plasma membrane. Hydrolysis of PIP_2 can inhibit Kir channels, provided that levels become limiting for channel activity. The level of inhibition depends on the strength of interaction of the channel with PIP_2 (Kobrinsky et al., 2000; H.Z., C.M.B.L., D.E.L., T. Mirshahi, unpublished data). We showed that the decrease in PIP_2 interactions with coexpression of a disease mutant subunit is coupled to an increase in sensitivity to Gq/G11 stimulation. This leads to a further loss of channel function as a response to these stimuli, which would exacerbate the disease phenotype under certain conditions. We speculate that this could be the mechanism underlying the periodic paralysis observed in these patients. Moreover, an increase in sensitivity to stimulation could be a trigger for an increase in the severity of cardiac arrhythmias.

Other mutations associated with both Andersen's syndrome (G300V and E303K) and Bartter's syndrome (C49Y, I51T, A214V, L220F) allosterically affected channel-PIP_2 interactions. These residues are all located near identified PIP_2 binding sites (Figure 4A, marked in green). The decrease in channel-PIP_2 interactions presents a novel mechanism to explain the pathophysiology of these diseases. The understanding of the molecular mechanisms underlying channelopathies has very important implications for the treatment and management of disease. Given the large number and high expression levels of Kir channels together with the variety of functional roles they fulfill, PIP_2-dependent channelopathies might prove to be common and to be present in other as yet unidentified genetic defects linked to Kir channels.

Experimental Procedures

Molecular Biology

All cDNA constructs were subcloned into the pGEMHE plasmid vector and used as described. Oocytes coexpressed with M1 and Kir2.1 cRNAs were injected with a 5:1 (M1:Kir2.1) ratio. Point mutants were produced by Pfu mutagenesis with a Quickchange kit (Stratagene, La Jolla, CA). Sequences were confirmed by DNA sequencing. Tetrameric cDNAs were constructed in three steps, as described previously (Lu et al., 1999a). HA epitopes were introduced into Kir2.1 cDNA by sequential overlap extension PCR. The epitope (YPYDVPDYA) was inserted at position 117 of Kir2.1. cRNA was produced with T7 RNA polymerase from a kit (Ambion, Austin, TX). RNAs were electrophoresed on formaldehyde gels, and concentrations were estimated from two dilutions, using RNA markers (GIBCO) as a standard.

Surface Labeling of Oocytes

We engineered an HA tag to the extracellular loop between M1 and the P region of Kir2.1 (Zerangue et al., 1999). Oocytes were blocked for 30 min in ND96 with 1% bovine serum albumin (BSA) at 4°C, labeled with 0.35 μg/ml rat monoclonal anti-HA antibody (3F10; Boehringer Mannheim) (in 1% BSA for 40 min at 4°C), washed at 4°C, and incubated with HRP-coupled secondary antibody (goat anti-rat) (in 1% BSA for 30 min). Cells were washed (1% BSA, 4°C) and transferred to ND96 without BSA. Individual oocytes were placed in 55 μl SuperSignal ELISA (Pierce) and incubated at room temperature for 1 min. Chemiluminescence was quantitated in a TD-20E luminometer (Turner Designs, CA). As a control, we used uninjected oocytes.

Electrophysiology

Oocytes were isolated from *Xenopus laevis* frogs (Nasco, Atkinson, WI) and injected with 0.1–4 ng of cRNA in 50 nl of sterile water. Currents were measured 1–5 days after injection. Macropatch currents were sampled at 4 KHz and filtered at 1 KHz; single-channel currents were sampled at 5–10 KHz and filtered at 1–2 KHz. Data were analyzed using either PCLAMP6 (macropaches) or pCLAMP8 (single-channel) software. Some of our own programs complemented this software package. Only those records containing no or few multiple open states were analyzed for fitting dwell time histograms. Records containing considerable numbers of multiple openings were only used to deduce parameters such as activity (NPo), mean open time (MTo), and opening frequency (Sui et al., 1998). The baseline drift was carefully removed before idealization with Fetchan. The mean open time is calculated as the weighted average of two time constants.

Electrodes contained 96 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES (pH 7.4) and had resistances of 0.3–0.8 MΩ (macropatches) and 1–20 MΩ (single-channel). Oocytes were studied at room temperature with perfusion at 0.1–1 ml/min using FVPP solution (96 mM KCl, 5 mM EDTA, 10 mM HEPES, 5 mM NaF, 3 mM Na_3VO_4, 10 mM Na_2PO_7 [pH 7.4 with NaOH]), which retarded hydrolysis of PIP_2 and thus stabilized currents (Huang et al., 1998). For on-cell single-channel recordings, the bath solution included 96 mM KCl, 5 mM EGTA, and 10 mM HEPES (pH 7.40). 100 μM gadolinium was routinely included in the pipette solution to suppress native stretch channel activity in the oocyte membrane.

Whole-oocyte currents were measured by conventional two-microelectrode voltage clamp. Agarose-cushion microelectrodes were used with resistances of 0.1–1.0 MΩ. Oocytes were constantly perfused with a high-potassium solution containing 91 mM KCl, 1 mM NaCl, 1 mM $MgCl_2$, 5 mM KOH/HEPES (pH 7.4). Current amplitudes were measured at −80 mV.

Error bars in the figures represent SEM. Each experiment shown or described was performed on three to five oocytes of the same batch. A minimum of two to three batches of oocytes was tested for each experiment shown. When the wild-type control population was compared with several mutant populations, an analysis of variance (ANOVA) was performed, and Dunnett's test was used as a post hoc test for individual mutants (<0.99 confidence interval). For other cases, the unpaired t test was used to assess statistical significance.

MTS reagents (Toronto Reasearch Chemicals) were stored at −20°C and were dissolved in bath solution prior to each experiment. MTSEA (2.5 mM), MTSET (2 mM), and MTSES (10 mM) were applied for 120 s and washed out for at least 100 s before polylysine was applied. Polylysine (300 μg/ml) with an average molecular weight of 7 KDa was used. DiC_8PIP_2 was purchased from Echelon Research Laboratories (Salt Lake City, UT). $DiC_8PI(4,5)P_2$ was dissolved in bath solution to the indicated concentrations. PIP_2 antibody (Assay Technologies, Ann Arbor, MI) was diluted 1:50 in FVPP solutions.

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